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Original Article

(–)Doxazosin is a necessary component for the hypotensive effect of (±)doxazosin during long-term administration in conscious rats

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Aim: Doxazosin is a racemic mixture of (–)doxazosin and (+)doxazosin that is currently used as an add-on therapy for hypertension. In this study we investigated the contribution of each enantiomer to the hypotensive action of long-term administration of (±)doxazosin in conscious rats.

Methods: Blood pressure of conscious SD rats was measured using a volume pressure recording system. The rats were orally administered (–)doxazosin, (+)doxazosin, or (±)doxazosin (8 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) for 12 weeks. Plasma concentrations of the agents were analyzed with HPLC. The effect of the agents on α_1-adrenoceptor was examined in isolated rat caudal artery preparations.

Results: Treatment of conscious rats with a single dose of (±)doxazosin (8 mg/kg) did not affected DBP and MBP, but significantly decreased SBP by 11.9% 4 h after the administration. Long-term treatment of conscious rats with (±)doxazosin significantly decreased SBP, DBP and MBP with a maximal decrease of SBP by 29.3% 8 h after the last administration. The rank order of the hypotensive actions caused by long-term treatment in conscious rats was (±)doxazosin>(+)doxazosin>>(–)doxazosin. However, the pK_B values for inhibiting NA-induced contraction of isolated rat caudal artery were (+)doxazosin (8.995)>(±)doxazosin (8.694)>(–)doxazosin (8.032). The plasma concentrations of (–)doxazosin, (+)doxazosin, and (±)doxazosin were 18.26±3.55, 177.11±20.66, and 113.18±13.21 ng/mL, respectively, 8 h after the last administration of these agents.

Conclusion: Long-term treatment with (±)doxazosin produces potent hypotensive action in conscious rats that seems to result from synergic interaction of the two enantiomers.

Keywords: doxazosin; enantiomer; blood pressure; hypotension; plasma drug concentration; α_1-adrenoceptor; noradrenaline; tail artery; conscious rat

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Introduction

Because a satisfactory blood pressure response is rarely reached with monotherapy alone in hypertensive patients, α_1-adrenoceptor antagonists are widely used as add-on drugs in combination therapy to achieve target blood pressures[1]. Data from an observational analysis of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) showed that the (±)doxazosin gastrointestinal therapeutic system was used as third-line therapy to lower blood pressure and cause a modest reduction in serum lipids[2]. In contrast to earlier findings in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial[3], the (±)doxazosin used in the ASCOT was not associated with an increased incidence of heart failure[2]. In addition, α_1-adrenoceptor antagonists are used worldwidely to relieve obstructive urinary symptoms, especially in men with benign prostatic hyperplasia (BPH), as many guidelines recommend them as first-line therapy for moderate to severe lower urinary tract symptoms (LUTS) suggestive of BPH.

The latest version of the guidelines on the management of BPH from the American Urological Association[4] and the guidelines on the management of male LUTS from the European Urological Association[5] refer to alfuzosin, doxazosin, tamsulosin and terazosin, while the Japanese guidelines for BPH also recommend naftopidil and silodosin[6]. The reports submitted to the US Food and Drug Administration Adverse Event Reporting System between 1997 and 2011 assessed the safety profiles of alfuzosin, doxazosin, tamsulosin, terazosin, naftopidil, silodosin and urapidil[7]. The total number of reports used was 1260182, but there were not enough naftopidil, silodosin and urapidil reports to compare those

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agents with the other four antagonists. Signal scores for ejaculation dysfunction were higher for tamsulosin treatment, and those for dizziness/vertigo were lower for doxazosin treatment than for alfuzosin, tamsulosin and terazosin[7].

Because of a chiral carbon in the doxazosin structure, it has two enantiomers: (–)doxazosin and (+)doxazosin. (±)Doxazosin and its enantiomers all are proved to be potent antagonists with balanced activity across the cloned human α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor subtypes expressed in rat-1 fibroblast cell lines[8]. However, we recently found that (–)doxazosin treatment decreased the carotid blood pressure to an extend less than those induced by (+)doxazosin and (±)doxazosin treatment in anesthetized rats, but its effect on the vesical micturition pressure was similar to (+)doxazosin and (±)doxazosin treatment in anesthetized rats and guinea pigs[9, 10]. These results indicated the chiral recognition of (+)doxazosin and (–)doxazosin in an intact biological system.

We further reported that (–)doxazosin, (+)doxazosin and (±)doxazosin treatment antagonized noradrenaline (NA)-induced vasoconstriction via α_1-adrenoceptors in a competitive manner in the rat thoracic aortic ring preparation, and the pA_2 value for (–)doxazosin treatment was markedly less than those for (+)doxazosin and (±)doxazosin[11]. Similar results were confirmed in the isolated common carotid, ear, mesenteric, pulmonary arteries and the thoracic aorta of rabbits[12, 13]. However, the contributions of (–)doxazosin and (+)doxazosin to the hypotensive effect of (±)doxazosin for long-term administration have not yet been examined in conscious animals.

In recent years, a new technique for measuring blood pressure in conscious rats using a volume pressure recording system has been validated in a number of studies[14, 15]. Long-term oral administration of (±)doxazosin (8 mg/kg for 12 weeks) in the rat was also reported in a study by Yono *et al*[16]. Therefore, the aims of the present study were (1) to compare the hypotensive effect of (±)doxazosin between a single oral administration and long-term oral administration in conscious rats and (2) to clarify the roles of (–)doxazosin and (+)doxazosin in the hypotensive effect of long-term oral administration of (±)doxazosin in conscious rats. In addition, we measured the plasma drug concentrations, which corresponded to the maximal hypotensive responses to the long-term administration of (–)doxazosin, (+)doxazosin and (±)doxazosin, and investigated the α_1-adrenoceptor blocking activity of (–)doxazosin, (+)doxazosin and (±)doxazosin against NA-induced contractile responses in the isolated caudal artery of the rat.

Materials and methods

Animals

Healthy 10-week-old, male, specific pathogen-free Sprague-Dawley (SD) rats weighing 300–320 g were included in this study and provided by the Experimental Animal Center of Hebei Province (Shijiazhuang, China). The animals were housed in a temperature (23±1 °C)- and humidity (50%±5%)-controlled room with a constant 12-h light/dark cycle (lights on from 08:00 to 20:00) and had free access to standard lab chow and tap water. Animals were allowed to habituate to the animal maintenance facilities for a period of at least 7 d before the beginning of the experiments. All animals were handled in accordance with our institute's guidelines for animal care and the NIH's Guide for the Care and Use of Laboratory Animals (2011). The present study was approved by the Hebei Medical University Ethics Committee for Animals.

Chemicals

(±)Doxazosin mesylate, (+)doxazosin mesylate and (–)doxazosin mesylate with a purity higher than 99.9% were synthesized and provided by the New Drug Research and Development Center of the North China Pharmaceutical Group Corporation (Shijiazhuang, China). (±)Doxazosin and its enantiomers were dissolved in ultrapure water to a concentration of 0.8 mg/mL. Yohimbine hydrochloride, propranolol hydrochloride, deoxycorticosterone acetate, desmethylimipramine hydrochloride, acetylcholine hydrochloride and (–)-noradrenaline bitartrate were purchased from Sigma Aldrich (St Louis, MO, USA). Heparin and sodium pentobarbital were purchased from Tianjin Biochemical Pharmaceutical Co, Ltd (Tianjin, China) and the Tianjin Yongda Chemical Reagent Development Centre (Tianjin, China), respectively. Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade, whereas all the other chemicals were of analytical reagent grade. HPLC-grade water was obtained from an ultrapure water system (Thermo Fisher Scientific, Waltham, MA, USA).

Experimental design

For the first set of experiments, 40 rats were randomly divided into four groups with 10 rats each. Group I served as a solvent control, and the rats in groups II, III and IV were administered orally 8 mg/kg of (–)doxazosin, (+)doxazosin or (±)doxazosin once daily for 12 weeks, respectively. During the 11th week of drug administration, blood pressure was measured for 12 h by the tail cuff method in the 40 conscious and nonfasted rats. The time required to complete the measurements in the 40 animals was 5 d, as measurements were done in only two rats randomly selected from each group every day. The rats were housed 5 per cage for the duration of the experiment, and food intake and weight gains were recorded once a week for the 12 weeks of treatment. On the last day of drug administration, blood samples were collected from the abdominal aorta 8 h after the last administration in rats anesthetized with pentobarbital (50 mg/kg, ip). Because the blood samples were used to determine the serum biochemistry and serum lipids, the rats were deprived of food for 12 h before collecting the blood. The blood samples were also used to determine the concentrations of (–)doxazosin, (+)doxazosin, and (±)doxazosin.

To clarify the possible influence of the fasting state on the plasma drug concentration, a second set of experiments was performed in 8 rats, which were randomly divided into fasted and nonfasted groups. A difference in the drug concentration-time curves in the plasma between fasted and nonfasted rats was studied. Approximately 0.5 mL blood was collected into heparinized Eppendorf tubes from the postorbital vein plexus of the rats at 0, 0.5, 1, 2, 4, 6, 8, and 12 h after a single oral

administration of 8 mg/kg (±)doxazosin. Additionally, time-dependent hypotensive effects and the maximum hypotensive effect induced by a single oral administration of 8 mg/kg (±)doxazosin were studied. Twelve rats were randomly divided into two groups, with one group receiving (±)doxazosin and the other group receiving solvent. Hypotensive responses were measured at 0, 2, 4, 8, and 12 h after a single oral administration of 8 mg/kg (±)doxazosin in conscious and nonfasted rats.

The last experiment was designed to determine the pA_2 or pK_B values for (–)doxazosin, (+)doxazosin and (±)doxazosin against NA-induced contraction via α_1-adrenoceptors in the rat isolated caudal artery.

Blood pressure measurement

Blood pressure was measured using a volume pressure recording (VPR) system (CODA 2, Kent Scientific, Torrington, CT, USA). The principle of the VPR method is similar to tail cuff inflation, but the VPR system uses two tail cuffs. The proximal occlusion cuff (O-cuff) constricts the tail artery while the distal VPR cuff measures the change in tail artery volume when blood flow resumes as the O-cuff deflates. This system has been validated in a number of studies[14, 15, 17]. These measurements were performed in a room maintained at 31 °C so as to ensure adequate blood flow through the tail to improve the signal at the VPR transducer. The rat was prewarmed in the room for 15 min and then placed in a restraining holder for 10 min before measurement. At least 10 consecutive cycles (inflation/deflation) were run for data collection. Additional cycles were conducted if insufficient data were obtained. The mean value of 4 to 8 similar cycles was used to determine experimental parameters, which included the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), heart rate (HR), tail blood flow and tail blood volume. Before the start of the blood pressure recordings, the rats were habituated to the measurements taken over two 10-min sessions each day for 3 d until stable recordings were obtained.

Determination of plasma drug concentrations

HPLC system and operating conditions

Except for the columns, the HPLC system employed for the achiral and chiral analyses was the same and consisted of the following components of the Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA): a quaternary solvent delivery system with an on-line degasser, an autosampler set at a sample volume of 10 μL, an Agilent column oven set at 30 °C and a fluorescence detector with λ_{ex} set at 255 and λ_{em} set at 385 nm. The columns used for analysis were a Zobax RP-C18 analytical column (150 mm×4.6 mm *id*, 5 μm; Agilent) and an Ultron ES-OVM column (150 mm×4.6 mm *id*, 5 μm; Shinwa, Kyoto, Japan) with an Ultron ES-OVM cartridge (10 mm×4.0 mm, 5 μm) for the achiral and chiral analyses, respectively. The HPLC separations were carried out using a phosphate buffer (30 mmol/L, pH 3.18): methanol (48:52, *v/v*) solution at a flow rate of 1 mL/min for the achiral analysis, and a phosphate buffer (20 mmol/L, pH 5.32): acetonitrile (86:14, *v/v*) solution at a flow rate of 0.8 mL/min for the chiral analysis.

Plasma sample preparation

Plasma samples (200 μL) were accurately measured and transferred into new 1.5 mL microcentrifuge tubes, and then 20 μL of the internal standard solution (prazosin, 0.8 μg/mL in water) was added. The mixture was extracted with 900 μL of hexane/ethyl acetate (1:1, *v/v*) by vortexing for 2 min. The organic and aqueous phases were separated by centrifugation at 6000 rounds per minute for 4 min. The organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dry residue was redissolved with 200 μL of mobile phase phosphate buffer (20 mmol/L, pH 5.32), vortex-mixed for 2 min and centrifuged at 12000 rounds per minute for 10 min. The supernatant was transferred into amber microvials, capped and placed in the autosampler.

Data collection and calculation

All data were collected using the Agilent ChemStation Software (Rev B.04.03). The concentrations of (±)doxazosin, (–)doxazosin and (+)doxazosin in the plasma samples were calculated by the corresponding calibration curves, which were constructed using a method published previously[18].

Serum biochemical analyses

Blood samples in non-anticoagulant tubes (Hubei Jinxing Science & Technology Development Co, Ltd, Wuhan, China) were left to clot at room temperature for 1 h, and then centrifuged at 3000 rounds per minute for 10 min. The serum was removed and kept at -20 °C until ready for use. Biochemical analyses were conducted with an Olympus AU5400 fully automatic biochemical analyzer (Olympus Corp, Tokyo, Japan). The levels of serum urea (UREA), creatinine (CRTN), alanine aminotransferase (ALT), total protein (TP), albumin (ALB), globulin (GLO, calculated by subtracting ALB from TP), albumin/globulin (A/G) ratio, aspartate aminotransferase (AST), creatine kinase (CK), total cholesterol (T-CHO), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) were determined using commercial kits from Simes Sikma (Beijing, China). Uric acid (UA) levels were determined using a commercial kit from Biosino Biotechnology and Science Inc (Beijing, China). Glucose (GLU) levels were determined using a commercial kit from Ningbo Rui Biotechnology Co, Ltd (Ningbo, China).

Isolated caudal artery

Rats anesthetized with a subcutaneous injection of urethane (1.5 g/kg) were euthanized by arterial exsanguination. The caudal artery was surgically exposed from the ventral side, then dissected from surrounding tissues and removed. The vascular endothelium was removed by gently rubbing the lumen with a scored polythene cannula, the external diameter of which was slightly smaller than the internal diameter of the blood vessel. A ring segment (4 mm long) without the endo-

thelium was mounted horizontally in a 10-mL organ bath by carefully inserting a tungsten wire through the lumen of the arterial ring preparation and anchoring it to a stationary support. Another similarly inserted tungsten wire was connected to an isometric tension transducer coupled to a polygraph (ERT-884, Youlin Electron Co, Kaifeng, China)[19] to record the change in tension of the preparation. A preload of 0.75 g was applied to the arterial ring preparations, and the preparations were allowed to equilibrate for 1 h in Krebs-Henseleit (K-H) solution with the following composition (mmol/L)[19, 20]: NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8, and $CaCl_2$ 2.52 with a pH 7.4. The K-H solution was maintained at 37 °C and aerated with 95% O_2 and 5% CO_2. Endothelium-denuded arteries were used only if acetylcholine (1 μmol/L) was unable to relax the arteries by >10% in the preparations precontracted with 0.3 μmol/L NA[21].

Desmethylimipramine (0.1 μmol/L), deoxycorticosterone (5 μmol/L), yohimbine (0.3 μmol/L), and propranolol (1 μmol/L) were added to the bath solution to block neuronal and extra neuronal uptake of NA and to block α_2-adrenoceptors and β-adrenoceptors, respectively[22]. Cumulative concentration-response curves to NA stimulation were constructed five times at 45-min intervals in each of the arterial rings tested, with the second set of concentration-response curves used as a control in the present study. (±)Doxazosin and (+)doxazosin (0.001, 0.01, and 0.1 μmol/L) as well as (–)doxazosin (0.003, 0.03, and 0.3 μmol/L) were added to the organ bath 20 min before the third, fourth and fifth concentration-response curves to NA treatment. One preparation was only treated with one of the three antagonists. Solvent instead of the antagonist was given to the arterial preparations in the control group. Vasoconstriction was expressed as a percentage of the maximum control response to NA.

Statistical analyses

Values are presented as the mean±SEM. A one-way ANOVA followed by a Dunnett's *post-hoc* test was used to analyze time-dependent hypotensive responses to (±)doxazosin and its enantiomers. A two-way ANOVA followed by a Bonferroni's *post-hoc* test was used to evaluate any differences between two sets of time-dependent hypotensive response curves, two sets of plasma drug concentration-time curves, or two data sets of the time-dependent changes in body weight. Statistical significance of the difference between two groups was determined by Student's *t*-test. In the isolated artery experiments, a comparison among three or more groups was made with a Dunnett's multiple comparison test. The EC_{50} values (molar concentration of the agonist that produced 50% of the maximal response) and E_{max} values (the maximal response) for agonists were calculated by nonlinear regression analysis using GraphPad Prism 5.00 software (GraphPad Software Inc, San Diego, CA, USA). A Schild analysis was performed with GraphPad Prism software to determine the pA_2 or pK_B values and the Schild plot slope for antagonists. A *P* value less than 0.05 was considered statistically significant. The data were analyzed using GraphPad Prism software.

Results

Changes in blood pressure and heart rate in nonfasted conscious rats after administration of a single dose of (±)doxazosin

The SBP, DBP, MBP, and HR did not change significantly 2, 4, 8, and 12 h after solvent administration in control rats ($P>0.05$, Figure 1). The SBP in rats treated with 8 mg/kg (±)doxazosin significantly decreased 4 h (113.21±3.63 mmHg) after administration compared with the pre-drug baseline level (128.54±5.98 mmHg; $P<0.05$), but there was no significant difference between each individual datum (racemic doxazosin group) and its respective control value (solvent group, Figure 1). A single administration of (±)doxazosin did not significantly affect the HR ($P>0.05$), and the average values of HR were 389.43±23.31, 343.02±14.06, 359.16±17.18, 397.77±25.53, and 344.99±9.23 (bpm) before and 2, 4, 8, and 12 h after (±)doxazosin administration, respectively.

Plasma concentration of (±)doxazosin in fasted and nonfasted conscious rats after administration of a single dose of (±)doxazosin

The limit of quantitation for (±)doxazosin was 5 ng/mL. The plasma (±)doxazosin concentration quickly increased to 254.61±27.69 ng/mL at 0.5 h, and then reached its peak level (306.97±43.47 ng/mL) 1 h after oral administration in fasted rats (Figure 2). The plasma (±)doxazosin concentration was

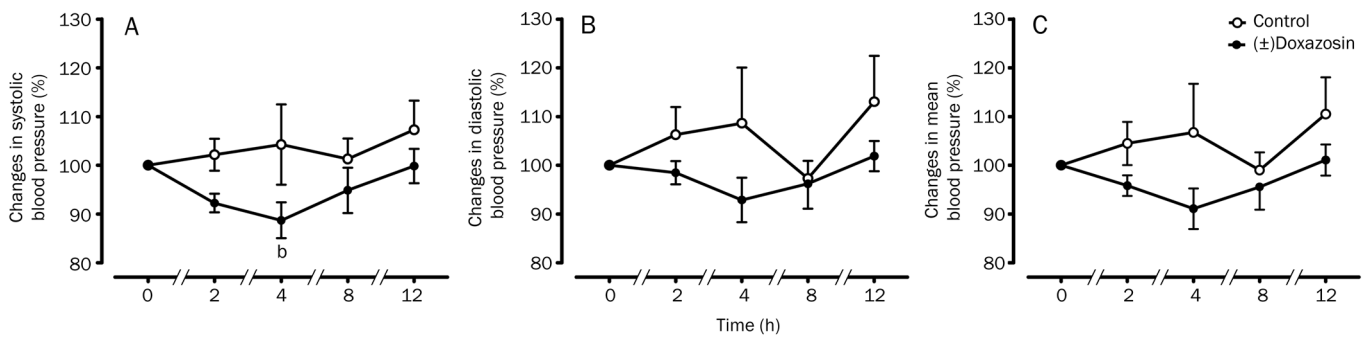

Figure 1. Changes in systolic blood pressure (A), diastolic blood pressure (B) and mean blood pressure (C) in nonfasted conscious rats after a single administration of 8 mg/kg (±)doxazosin. The data are expressed as the mean±SEM. $n=6$. $^bP<0.05$ *vs* baseline.

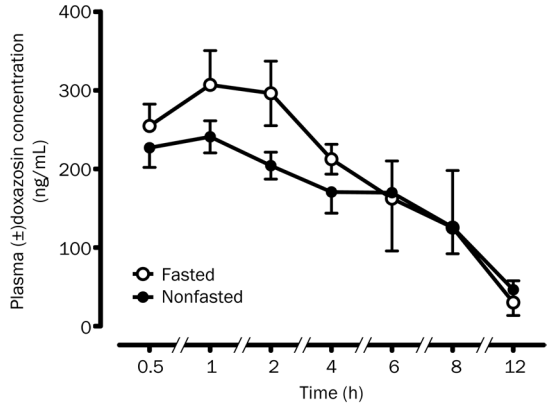

Figure 2. The plasma concentration of (±)doxazosin after a single administration of 8 mg/kg (±)doxazosin in fasted and nonfasted conscious rats. The data are expressed as the mean±SEM. *n*=4.

maintained at a high level (296.10±40.97 ng/mL) at 2 h, and decreased to 30.22±16.53 ng/mL 12 h after administration in fasted rats (Figure 2). Although the plasma concentration of (±)doxazosin in nonfasted rats was slightly lower than that in fasted rats 0.5, 1, and 2 h after oral administration of the same dose, the drug concentration-time curve for (±)doxazosin in fasted rats was not significantly different from that in nonfasted rats (P>0.05, Figure 2). In addition, stable (±)doxazosin concentrations were maintained at 4 h (170.75±27.05 ng/mL) and 6 h (169.87±40.29 ng/mL) after oral administration in nonfasted rats.

Effects of long-term administration of (–)doxazosin, (+)doxazosin, and (±)doxazosin on blood pressure and heart rate in nonfasted conscious rats

The SBP, DBP, MBP, and HR did not change significantly 2, 4, 8, and 12 h after solvent administration in control rats (P>0.05, Figure 3). In the rats treated with long-term administration of (±)doxazosin for 12 weeks, the SBP significantly decreased 2 (123.03±3.63 mmHg), 4 (123.93±6.53 mmHg), 8 (102.15±3.68 mmHg), and 12 h (113.86±5.28 mmHg) after the last administration of 8 mg/kg (±)doxazosin compared with the pre-drug baseline level (144.57±4.18 mmHg; P<0.01, Figure 3). The hypotensive effects on DBP and MBP of long-term administration of (±)doxazosin were similar to the effects on SBP. Statistical analysis indicated significant differences in the hypotensive effects on SBP, DBP, and MBP by (±)doxazosin between the solvent group and the (±)doxazosin group (Figure 3). (+)Doxazosin treatment had similar hypotensive effects as (±)doxazosin treatment; however, the maximal decreases (8 h after the last administration) in SBP, DBP, and MBP induced by (+)doxazosin treatment were significantly smaller than the decreases induced by (±)doxazosin treatment at the same dose (P<0.05, Figure 4). Long-term administration of (–)doxazosin produced significant, but mild, hypotensive effects on the SBP (112.67±6.56 mmHg), DBP (72.34±4.24 mmHg), and MBP (85.46±4.96 mmHg) 8 h after the last administration compared with the pre-drug baseline levels (132.81±4.83, 87.15±3.58, and 102.00±3.96 mmHg) (P<0.05 and 0.01; Figure 3). However, the maximal decreases (8 h after the last administration) in SBP, DBP and MBP induced by (–)doxazosin treatment were not significantly different from those induced by solvent (P>0.05, Figure 4).

Before long-term administration, the HR values in the solvent, (–)doxazosin, (+)doxazosin and (±)doxazosin groups were 386.11±15.45, 385.14±16.12, 392.91±17.12, and 365.85±21.06 (bpm, P>0.05), respectively, and long-term administration of (–)doxazosin, (+)doxazosin, or (±)doxazosin did not significantly affect the HR compared with the pre-drug baseline levels (P>0.05, n=8-10, data not shown). However, statistical analysis determined a significant positive chronotropic effect 8 (336.61±16.57 bpm *vs* 427.43±34.4 bpm) and 12 h (332.24±22.68 bpm *vs* 435.15±25.85 bpm) after the last administration of (±)doxazosin (P<0.05 and 0.01) in the (±)doxazosin group compared with the solvent group.

Plasma drug concentrations in rats administered orally with (–)doxazosin, (+)doxazosin, or (±)doxazosin for 12 weeks

Plasma drug concentrations were measured 8 h after the

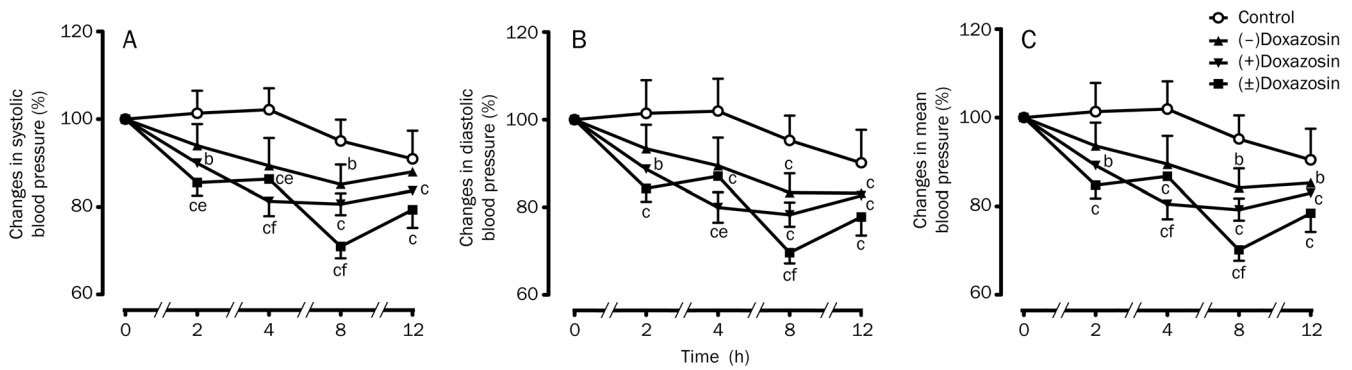

Figure 3. Changes in systolic (A), diastolic (B) and mean blood pressure (C) in nonfasted conscious rats after the long-term administration of (–)doxazosin, (+)doxazosin, and (±)doxazosin (8 mg/kg, once daily for 12 weeks, respectively). The data are expressed as the mean±SEM. *n*=10. [b]P<0.05, [c]P<0.01 *vs* baseline. [e]P<0.05, [f]P<0.01 *vs* control.

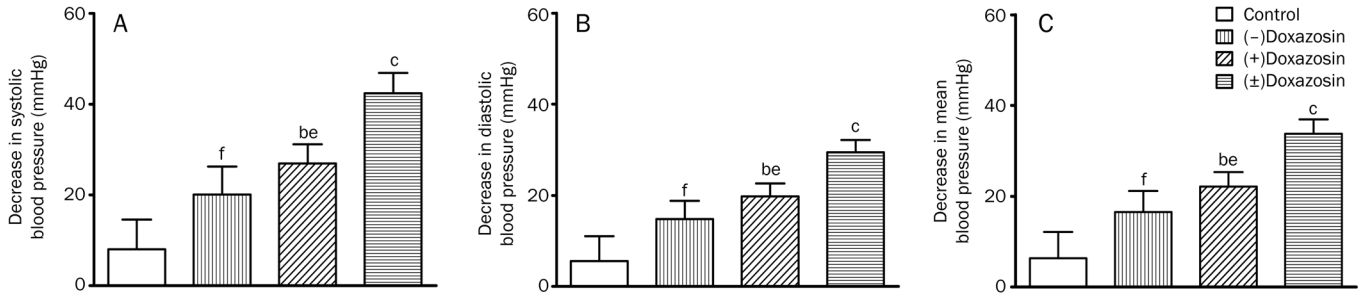

Figure 4. Decreases in the systolic (A), diastolic (B) and mean blood pressure (C) 8 h after the last administration of (–)doxazosin, (+)doxazosin, and (±)doxazosin. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* (±)doxazosin.

last administration of (–)doxazosin, (+)doxazosin, and (±)doxazosin (Figure 5) and were 18.26±3.55, 177.11±20.66, and 113.18±13.21 ng/mL, respectively. The concentrations of (–)doxazosin and (±)doxazosin were significantly lower than the (+)doxazosin concentration ($P<0.05$ and 0.01, Figure 6), and the concentration ratio of (–)doxazosin/(+)doxazosin was 0.10. A chiral separation of (±)doxazosin-containing plasma samples was performed, and the (–)doxazosin and (+)doxazosin components were 12.01±2.45 and 96.56±9.94 ng/mL, respectively. The concentration ratio of (–)doxazosin/(+)doxazosin in the plasma from rats administered orally with (±)doxazosin was 0.12.

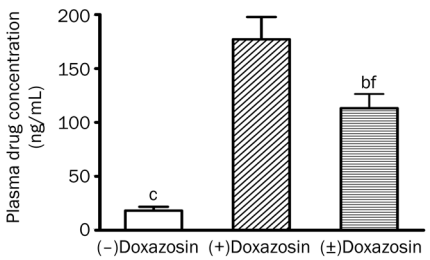

Figure 6. Plasma concentrations of (–)doxazosin, (+)doxazosin, and (±)doxazosin 8 h after the last administration of (–)doxazosin, (+)doxazosin, and (±)doxazosin. Data are expressed as the mean±SEM. $n=10$. $^{b}P<0.05$, $^{c}P<0.01$ *vs* (+)doxazosin. $^{f}P<0.01$ *vs* (–)doxazosin.

Effects of long-term administration of (–)doxazosin, (+)doxazosin and (±)doxazosin on biochemical parameters and the body weight of the rat

Table 1 shows the results of the serum biochemical analyses after long-term administration of (–)doxazosin, (+)doxazosin and (±)doxazosin. The three agents produced no significant changes in liver function (ALT, AST, TP, ALB, GLO, and A/G) or kidney function (UREA, UA, and CRTN). The levels of CK and GLU were also not changed by the agents. However, long-term treatment with (+)doxazosin significantly increased the HDL-C level, and long-term treatment with (–)doxazosin significantly decreased the LDL-C level ($P< 0.01$, Table 1).

Before long-term oral administration of solvent, (–)doxazosin, (+)doxazosin, and (±)doxazosin, the average body weights of rats in the four groups were not significantly different (359.9±6.98, 361.9±4.23, 359.4±6.30, and 357.4±4.61 g, respectively; $n=10$) ($P>0.05$). The average body weights of rats in the solvent group at the end of the 3rd, 6th, 9th and 12th week (449.2±12.38, 489.8±14.55, 516±14.04, and 539.7±14.69 g, respectively) were significantly greater than that (359.9±6.98 g) before administration of the solvent ($P<0.01$, $n=10$). Long-term administration of (–)doxazosin, (+)doxazosin, and (±)doxazosin for 12 weeks did not significantly affect the body weight gain of the rat compared with long-term solvent administration ($P>0.05$, $n=10$, data not shown).

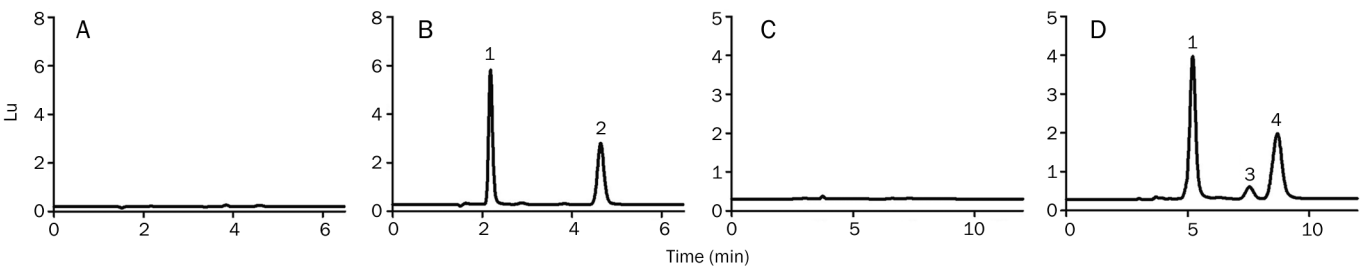

Figure 5. Typical HPLC chromatograms obtained from a blank plasma sample determined using an achiral column (A); a rat plasma sample collected after the oral administration of a racemic doxazosin and determined using an achiral column (B); a blank plasma sample determined using a chiral column (C); the rat plasma sample aliquoted from that for (B) and determined by a chiral column (D). Peaks: 1) prazosin, 2) doxazosin, 3) (–)doxazosin, and 4) (+)doxazosin. For more experimental details, refer to the Materials and methods section.

Table 1. Biochemical parameters of the rats treated with (-)doxazosin, (+)doxazosin, or (±)doxazosin at 8 mg·kg^{-1}·d^{-1} for 12 weeks. $^{c}P<0.01$ *vs* control. $n=10$.

	Control	(−)Doxazosin	(+)Doxazosin	(±)Doxazosin
UREA (mmol/L)	5.28±0.27	4.90±0.13	5.43±0.19	4.54±0.24
CRTN (μmol/L)	33.20±1.15	28.80±1.21	33.60±1.57	31.00±1.95
UA (μmol/L)	70.80±9.31	61.50±5.75	58.70±6.13	58.30±8.19
ALT (U/L)	36.30±3.56	38.50±4.58	36.10±3.18	34.80±3.23
AST (U/L)	154.30±19.42	151.30±17.32	140.00±18.51	150.50±17.52
TP (g/L)	57.31±1.71	56.66±1.05	57.57±0.66	58.28±1.21
ALB (g/L)	28.67±0.77	28.84±0.81	28.78±0.42	29.16±0.43
GLO (g/L)	28.64±1.06	27.82±0.50	28.79±0.41	29.12±0.88
A/G (ratio)	1.01±0.02	1.03±0.03	1.00±0.02	1.01±0.02
CK (U/L)	800.70±139.70	889.70±205.30	656.00±96.34	937.10±201.50
GLU (mmol/L)	5.84±0.38	5.81±0.29	5.90±0.38	5.33±0.32
T-CHO (mmol/L)	1.15±0.04	1.11±0.06	1.20±0.04	1.13±0.07
TG (mmol/L)	0.37±0.05	0.27±0.04	0.31±0.04	0.35±0.05
HDL-C (mmol/L)	0.33±0.01	0.34±0.01	0.39±0.01^{c}	0.36±0.01
VLDL-C (mmol/L)	0.17±0.02	0.12±0.02	0.14±0.02	0.16±0.02
LDL-C (mmol/L)	0.24±0.02	0.15±0.02^{c}	0.23±0.02	0.18±0.01

Effects of (−)doxazosin, (+)doxazosin, and (±)doxazosin treatment on the NA-induced contractile response in the isolated caudal artery of the rat

In the solvent control group, there were no significant differences in the E_{max} values in the 2nd, 3rd, 4th, or 5th set of concentration-response curves to NA treatment (1.75±0.05, 1.76±0.07, 1.80±0.07, and 1.93±0.06 g, respectively; $P>0.05$). The EC_{50} values calculated from the four concentration-response curves to NA treatment were not significantly different from each other (data not shown). Before treatment with (−)doxazosin, (+)doxazosin, and (±)doxazosin, the E_{max} values (1.61±0.02, 1.84±0.06, and 1.77±0.13 g, respectively) or the -LogEC_{50} (mol/L) values (6.76±0.04, 6.78±0.10, and 7.02±0.23, respectively) obtained from the concentration-response curves to NA treatment were not significantly different from each other ($P>0.05$, $n=6$).

(−)Doxazosin (0.003, 0.03, and 0.3 μmol/L), (+)doxazosin and (±)doxazosin (0.001, 0.01 and 0.1 μmol/L) treatment produced a shift to the right in the concentration-response curves to NA treatment (Figure 7), but there were no significant changes in the E_{max} values ($P>0.05$, $n=6$, data not shown). The slope of the Schild plot for (−)doxazosin, (+)doxazosin or (±)doxazosin treatment (0.607±0.028, 0.647±0.018, or 0.716±0.028, respectively) was significantly different from unity ($P<0.05$), indicating that the three agents non-competitively inhibited the concentration-response curves to NA treatment in the isolated rat caudal artery. The pK_B value of (−)doxazosin (8.032±0.039) was significantly smaller ($P<0.01$) than the pK_B values of (+)doxazosin (8.995±0.032) and (±)doxazosin (8.694±0.032), and the pK_B value of (±)doxazosin was significantly smaller than the pK_B value of (+)doxazosin ($P<0.01$).

Discussion

The plasma concentration of (±)doxazosin reached the peak level (240.9±40.9 ng/mL) 1 h after a single oral dose of 8 mg/kg and decreased by 29.1% at 4 h (170.8±54.1 ng/mL) in

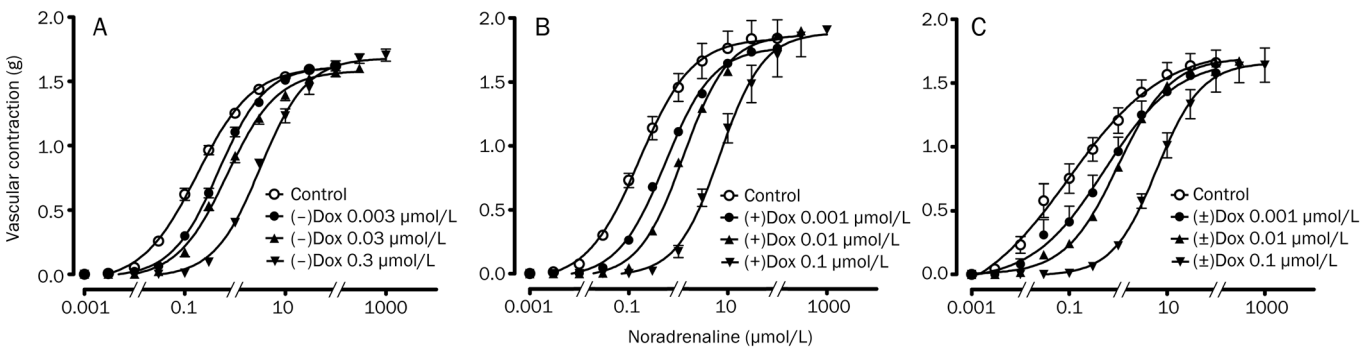

Figure 7. The effects of (−)doxazosin (A), (+)doxazosin (B), and (±)doxazosin (C) on the contractile responses to NA in the isolated caudal artery of the rat. Points represent the mean values and vertical bars show the SEM. $n=6$.

the nonfasted conscious rat. A single oral administration produced a significant decrease only in the SBP, with a maximal decrease of 11.9% 4 h after administration. However, a long-term (12-week) administration of 8 mg/kg (±)doxazosin significantly decreased the SBP, DBP, and MBP 2, 4, 8, and 12 h after administration of the last dose in the nonfasted conscious rat. The maximal hypotensive response to long-term administration of (±)doxazosin was observed 8 h after administration of the last dose with a decrease of 29.3% in SBP.

These results suggest that long-term administration of (±)doxazosin obviously alters not only its hypotensive activity but also the time to peak effect compared with a single dose administration. It is well known that doxazosin opposes the excitatory effects of NA released from sympathetic nerve endings at α_1-adrenoceptors, and causes dilation of the blood vessels, thereby reducing the BP. Thus, if hypotensive effects induced by (–)doxazosin, (+)doxazosin, and (±)doxazosin differ from each other in the study of long-term administration, it is very important to know the plasma concentrations of the three agents when the peak hypotensive effects are reached.

Previously, we reported that hypotensive responses to intravenously administered (–)doxazosin were significantly smaller than those to (+)doxazosin and (±)doxazosin and that (+)doxazosin treatment produced a larger decrease in the BP than (±)doxazosin treatment in acute experiments on anesthetized rats[9]. In the present study, long-term (12-week) administration of racemic doxazosin and its enantiomers to the conscious rat revealed unexpected results. Long-term administration of (–)doxazosin produced a small but statistically significant decrease in the SBP, DBP, and MBP 8 h after administration of the last dose compared with the pre-drug baseline levels (Figure 3). However, the maximal decrease in the BP induced by (–)doxazosin treatment at 8 h was not significantly different from that induced by solvent treatment (Figure 4). Although long-term administration of 8 mg/kg (±)doxazosin and (+)doxazosin significantly decreased the SBP, DBP, and MBP at 2, 4, 8, and 12 h in the nonfasted conscious rat, the maximal decreases in SBP, DBP, and MBP induced by (+)doxazosin treatment at 8 h were significantly smaller than those induced by (±)doxazosin treatment (Figure 4). Therefore, the rank order of the hypotensive responses to long-term oral administration in conscious rats was as follows: (±)doxazosin>(+)doxazosin>>(–)doxazosin.

NA produces vasoconstriction via α_1-adrenoceptors in the isolated caudal artery of the rat. We observed that (±)doxazosin and its enantiomers produced a shift to the right of the concentration-response curves to NA treatment with no significant changes in the E_{max} values. The pK_B value of (±)doxazosin treatment (8.694±0.032) was significantly smaller than that of (+)doxazosin treatment (8.995±0.032), but significantly larger than that of (–)doxazosin treatment (8.032±0.039). Previous studies also obtained similar results when observing the potency of (–)doxazosin, (+)doxazosin, and (±)doxazosin against NA-induced vasoconstriction via α_1-adrenoceptors in the isolated rat thoracic aorta[11] and mesenteric artery[23]. (±)Doxazosin is a racemic mixture containing equal amounts of two enantiomers, (–)doxazosin, and (+)doxazosin. Thus, the amount of either (+)doxazosin or (–)doxazosin contained in 8 mg/kg (±)doxazosin is equal to 4 mg/kg. Theoretically, a rank order of the hypotensive response to long-term oral administration of (±)doxazosin and its enantiomers should be (+)doxazosin>(±)doxazosin>(–)doxazosin, assuming that both (–)doxazosin and (+)doxazosin exhibit the same pharmacokinetic behavior in the rat.

Seeing that drug effectiveness increased in direct proportion to its plasma concentration, the plasma drug concentrations were measured 8 h (time to peak effect) after the last administration of (–)doxazosin, (+)doxazosin, and (±)doxazosin in the present study of long-term administration. The plasma concentrations of (–)doxazosin (18.26 ng/mL) and (±)doxazosin (113.18 ng/mL) were found to be much lower than the (+)doxazosin plasma concentration (177.11 ng/mL) in the rats that were orally administered with the three agents at 8 mg/kg for 12 weeks. These results clearly indicated the possibility that the clearance of (–)doxazosin from the blood might be much faster than that of (+)doxazosin because similar results were also obtained in rats treated with intravenously administered (–)doxazosin or (+)doxazosin (unpublished data).

A chiral separation of (±)doxazosin was further performed using the plasma samples from rats that received long-term administration of (±)doxazosin. The (–)doxazosin and (+)doxazosin components were 12.01±2.45 and 96.56±9.94 ng/mL, respectively, and the concentration ratio of (–)doxazosin/(+)doxazosin was 0.12. A similar ratio (0.10) was observed in rats treated with long-term administration of (–)doxazosin and (+)doxazosin at 8 mg/kg. These results suggest that there is no pronounced pharmacokinetic interactions between (–)doxazosin and (+)doxazosin in the rat. Hence, pharmacokinetic considerations could not explain the large difference in hypotensive responses between the long-term administration of (±)doxazosin and (+)doxazosin.

Generally speaking, the synergistic action in combination therapy comes from the actions of two components on the different targets. It has been reported that treatment with a combination of atenolol and nitrendipine possesses an obvious synergism on blood pressure reduction in hypertensive rats[24]. Aliskiren is a novel renin-angiotensin aldosterone system inhibitor, and the combination therapy of aliskiren and amlodipine provides a more effective blood pressure reduction than monotherapy with either drug alone in patients[25]. It is unclear whether other targets are involved in the synergistic action of (±)doxazosin. The responses to blocking the effect of (–)doxazosin on calcium channels, β-adrenoceptors or angiotensin II receptors remain to be identified in the near future.

The general health status of the animals was evaluated after 12-week administration of the three agents because a bad health status could affect the arterial blood pressure of the rat. Average body weight was determined for each of the four groups during long-term administration. The average body weight of control rat at 3, 6, 9, and 12 weeks of age was increased normally from 359.9±6.98 g (initial weight) to 449.2±12.38, 489.8±14.55, 516±14.04 and 539.7±14.69 g, respec-

tively. (–)Doxazosin, (+)doxazosin, and (±)doxazosin treatment did not significantly affect the body weight gain of the rat. In addition, the three agents did not significantly affect the liver or kidney function or the levels of CK and GLU in the rats. However, (+)doxazosin treatment significantly increased the HDL-C level while (–)doxazosin significantly decreased the LDL-C level, which was consistent with a previous report indicating that racemic doxazosin had beneficial effects on HDL-C and LDL-C levels in patients with hypertension and type 2 diabetes[26]. Therefore, the possible influence of the variation in health status caused by the long-term administrations on their hypotensive responses was excluded.

Because the blood samples used to assay the serum biochemistry and lipid profile were also used to analyze the plasma concentrations of (±)doxazosin and its enantiomers, the rats were deprived of food for 12 h before collecting the blood. Accordingly, an experiment was conducted to investigate the influence of food intake on the rat plasma concentration of (±)doxazosin because the hypotensive responses to long-term administration of the three agents were recorded in the non-fasting state. The results of this investigation indicated that there were no significant differences in the plasma concentration-time curves to (±)doxazosin treatment between fasted and nonfasted rats, suggesting that food deprivation might not affect the plasma concentrations of (–)doxazosin, (+)doxazosin, and (±)doxazosin at 8 h after each administration in the rats that received a 12-week drug treatment.

Considering the present data overall, we propose that (–)doxazosin obviously potentiated the hypotensive response to (+)doxazosin when 8 mg/kg (±)doxazosin was orally administered to the rat for 12 weeks. This hypothesis is supported by the following data: (1) The long-term administration of (–)doxazosin at 8 mg/kg was not capable of producing a significant decrease in the arterial blood pressure in conscious rats compared with the solvent control. (2) The maximal decrease in arterial blood pressure induced by long-term administration of 8 mg/kg (±)doxazosin was significantly larger than that induced by 8 mg/kg (+)doxazosin. (3) The plasma concentration of the (+)doxazosin component at the time of peak hypotensive response to long-term treatment with 8 mg/kg (±)doxazosin was 96.56±9.94 ng/mL, which was significantly lower than the plasma concentration of (+)doxazosin (177.11 ng/mL) in rats that received long-term treatment with 8 mg/kg (+)doxazosin. (4) Finally, a pronounced pharmacokinetic interaction between (–)doxazosin and (+)doxazosin and the potential long-term toxic effects induced by the three agents was shown to be negligible.

The mechanisms underlying this potentiation remain to be elucidated, but it appears that a pharmacodynamic interaction between (–)doxazosin and (+)doxazosin, rather than a pharmacokinetic interaction, is involved. The limited data of the present study also suggest that the antagonistic effects of doxazosin and its enantiomers directly on the α_1-adrenoceptors located in vascular beds are not solely responsible for the hypotensive response in the conscious rats.

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Author contribution

Lei-ming REN and De-zhi KONG designed and supervised the research; Jing ZHAO, Ya-qin ZHEN, and Qing LI performed the research and analyzed the data; Miao WANG, Yan ZHAO, and Dong-kai WANG helped with part of the research; and Jing ZHAO, De-zhi KONG, and Lei-ming REN wrote the manuscript.

References

1 Sever PS, Messerli FH. Hypertension management 2011: optimal combination therapy. Eur Heart J 2011; 32: 2499-506.
2 Chapman N, Chang CL, Dahlof B, Sever PS, Wedel H, Poulter NR, for the ASCOT Investigators. Effect of doxazosin gastrointestinal therapeutic system as third-line antihypertensive therapy on blood pressure and lipids in the Anglo-Scandinavian Cardiac Outcomes Trial. Circulation 2008; 118: 42–8.
3 ALLHAT Officers and Coordinators for the ALLHATT Collaborative Research Group. Diuretic versus α-blocker as first-step antihypertensive therapy. Final results from the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). Hypertension 2003; 42: 239–46.
4 McVary KT, Roehrborn CG, Avins AL, Barry MJ, Bruskewitz RC, Donnell RF, *et al*. Update on AUA guideline on the management of benign prostatic hyperplasia. J Urol 2011; 185: 1793–803.
5 Oelke M, Bachmann A, Descazeaud A, Emberton M, Gravas S, Michel MC, *et al*. Guidelines on the treatment of non-neurogenic male LUTS (2011). http://www.uroweb.org/gls/pdf/12_Male_ LUTS.pdf
6 Homma Y, Gotoh M, Yokoyama O, Masumori N, Kawauchi A, Yamanishi T, *et al*. Outline of JUA clinical guidelines for benign prostatic hyperplasia. Int J Urol 2011; 18: 741-56.
7 Yoshimura K, Kadoyama K, Sakaeda T, Sugino Y, Ogawa O, Okuno Y. A Survey of the FAERS database concerning the adverse event profiles of α_1-adrenoreceptor blockers for lower urinary tract symptoms. Int J Med Sci 2013; 10: 864–9.
8 Hatano A, Tang R, Walden PD, Lepor H. The α-adrenoceptor antagonist properties of the enantiomers of doxazosin in human prostate. Eur J Pharmacol 1996; 313: 135–43.
9 Ma SP, Ren LM, Zhao D, Zhu ZN, Wang M, Lu HG, *et al*. Chiral selective effects of doxazosin enantiomers on blood pressure and urinary bladder pressure in anesthetized rats. Acta Pharmacol Sin 2006; 27: 1423–30.
10 Tian HL, Ren LM. Effects of doxazosin enantiomers by different routes of administration on urinary bladder function in guinea pigs. Chin J Pharmacol Toxical 2007; 21: 118-23.
11 Zhao D, Duan LH, Wang FY, Wang M, Lu HG, Wu ZG, *et al*. Chiral recognition of doxazosin enantiomers in 3 targets for therapy as well as adverse drug reactions in animal experiments. Can J Physiol Pharmacol 2012; 90: 1623–33.
12 Niu C, Zhao D, Jia XM, Ren LM. α_1-Adrenoceptor antagonist profile of doxazosin and its enantiomers in isolated rabbit blood vessels. Chin J Pharmacol Toxicol 2003; 17: 354–9.
13 Lu HG, Liu LF, Ren LM, Zhao QH, Dun LH, Zhang XY. Effects of

doxazosin enantiomers on α-adrenoceptors of isolated rabbit blood vessels. Acta Pharm Sin 2007; 42: 145–51.

14 Aukes AM, Vitullo L, Zeeman GG, Cipolla MJ. Pregnancy prevents hypertensive remodeling and decreases myogenic reactivity in posterior cerebral arteries from Dahl salt-sensitive rats: a role in eclampsia? Am J Physiol Heart Circ Physiol 2007; 292: H1071–6.

15 Férézou-Viala J, Roy AF, Sérougne C, Gripois D, Parquet M, Bailleux V, *et al*. Long-term consequences of maternal high-fat feeding on hypothalamic leptin sensitivity and diet-induced obesity in the offspring. Am J Physiol Regulatory Integrative Comp Physiol 2007; 293: R1056–62.

16 Yono M, Foster HE Jr, Shin D, Takahashi W, Pouresmail M, Latifpour J. Doxazosin-induced up-regulation of α_{1A}-adrenoceptor mRNA in the rat lower urinary tract. Can J Physiol Pharmacol 2004; 82: 872-8.

17 Taylor PD, Khan IY, Lakasing L, Dekou V, O'Brien-Coker I, Mallet AI, *et al*. Uterine artery function in pregnant rats fed a diet supplemented with animal lard. Exp Physiol 2003; 88: 389-98.

18 Liu K, Zhong D, Chen X. Enantioselective determination of doxazosin in human plasma by liquid chromatography-tandem mass spectrometry using ovomucoid chiral stationary phase. J Chromatogr B Analyt Technol Biomed Life Sci 2010; 878: 2415–20.

19 Li L, Jia ZH, Chen C, Wei C, Han JK, Wu YL, *et al*. Physiological significance of P2X receptor-mediated vasoconstriction in five different types of arteries in rats. Purinergic Signal 2011; 7: 221–9.

20 Li L, Wu T, Wei C, Han JK, Jia ZH, Wu YL, *et al*. Exhaustive swimming differentially inhibits P2X1 receptor- and α_1-adrenoceptor-mediated vasoconstriction in isolated rat arteries. Acta Pharmcol Sin 2012; 33: 221–9.

21 Dora KA, Ings NT, Garland CJ. K_{Ca} channel blockers reveal hyperpolarization and relaxation to K^+ in rat isolated mesenteric artery. Am J Physiol Heart Circ Physiol 2002; 283: H606–14.

22 Muramatsu I, Ohmura T, Kigoshi S, Hashimoto S, Oshita M. Pharmacological subclassification of α-adrenoceptors in vascular smooth muscle. Br J Pharmacol 1990; 99: 197–201.

23 Wang M, Ren XJ, Zhao QH, Lin LX, Wang X, Zhao Y, *et al*. Relaxant and contractile responses of detrusor muscle strips obtained from bladder outlet obstructed rats treated with doxazosin enantiomers. Can J Physiol Pharmacol 2011; 89: 883–90.

24 Xie HH, Miao CY, Jiang YY, Su DF. Synergism of atenolol and nitrendipine on hemodynamic amelioration and organ protection in hypertensive rats. J Hypertens 2005; 23: 193–201.

25 Liu YK, Chen K, Kou X, Han Y, Zhou L, Zeng CY. Aliskiren and amlodipine in the management of essential hypertension: meta-analysis of randomized controlled trials. PLoS One 2013; 8: 1–10.

26 Glanz M, Garber AJ, Mancia G, Levenstein M. Meta-analysis of studies using selective α_1-blockers in patients with hypertension and type 2 diabetes. Int J Clin Pract 2001; 55: 694–701.